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A gene transfection for rat mesenchymal stromal cells in biodegradable gelatin scaffolds containing cationized polysaccharides

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Short / Running Title: Gene transfection of BMSC in 3D scaffolds

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Abstract

The objective of this study was to design three-dimensional scaffolds of bone marrow-derived stem cells capable of their gene transfection and evaluate the transfection extent. Three-dimensional scaffolds of gelatin and β-tricalcium phosphate (β-TCP) were prepared. Spermine-introduced pullulan (spermine-pullulan) was prepared as the non-viral carrier of gene transfection. The spermine-pullulan was mixed with a luciferase plasmid DNA to prepare their polyion complex. The scaffolds were treated with succcinylated gelatin (suc-gel) at different concentrations, treated in different methods of freeze-drying and dehydrothermal treatment, and placed in the aqueous of complexes to prepare various scaffolds containing the complex. When the stem cells were seeded into the scaffolds to evaluate the gene transfection of cells, the level of plasmid DNA transfection depended on the method of complex containing scaffolds preparation. The complexes were released with time from the scaffolds although the release profile depended on the type of scaffolds. The order of gene transfection for the stem cells in the scaffolds was in good accordance with that of plasmid DNA released. It is possible that cells were transfected with the complexes released from the scaffolds.

Keywords

Drug release; gelatin; genetic engineering; polysaccharide; scaffold; stem cells.

1. Introduction

Most of the current research work on stem cells is being performed in the culture system of two-dimension (2D) which is quite different from that of living body [1]. Needless to say, the natural extracellular environment of cells in the body is of three-dimensional (3D) structure. It has been generally recognized that cells are strongly influenced by their extracellular environment [2]. When compared between the 2D and 3D cell culture systems, the cell-cell and cell-substrate interactions must be different and consequently affect the property and biological activity of stem cells [2]. Taken together, it will be physiologically better to perform cell experiments in the 3D environment which is close to the natural in vivo condition of cells. Similarly, the gene transfection of cells will be influenced by the 3D culture.

Several researches of gene transfection have been reported for cells cultured in the 3D scaffolds of fibrin hydrogels [3], non-woven fabrics of polyethylene telephtalate (PET) [4, 5], and the sponges of synthetic polymers [6-8]. However, most of scaffold materials are generally prepared from non-degradable polymers.

Bone marrow mesenchymal stromal cells (BMSC) are one of the most important cells not only for basic researches of biology and medicine, but also for the clinical applications, because they have their own potentials to differentiate into various cell lineages, such as osteocytes [9], chondrocytes [10], adipocytes [11], dopamine-producing cells [12, 13], and tenocytes [14]. BMSC can be isolated from patients themselves and easily expanded in vitro, which is useful from the clinical viewpoint. In addition, BMSC have been widely used to make clear the mechanism of cell proliferation and differentiation [15, 16]. Gene transfection is one of the technologies necessary to actively proceed the basic cell researches. However, generally,

the efficiency of gene transfection is low for the BMSC compared with that of other cells. It is, therefore, necessary to develop the technology and methodology of gene transfection for stem cells.

In this study, a new technology of gene transfection for BMSC is introduced with the 3D scaffolds of sponge type prepared from biodegradable components. The biodegradable 3D scaffolds were prepared from gelatin and β-tricalcium phosphate (β-TCP). Gelatin is a denatured collagen and bioabsorbable, and has been clinically used. It is reported that the scaffold of gelatin could release growth factors (basic fibroblast growth factors [17], bone morphogenic protein-2 [18], transforming growth factor β1 [19]), nucleic acids (plasmid DNA [20] and small interfering RNA [21, 22]), and low-molecular-weight drugs [23, 24]. The combination of bioactive substances by use of gelatin is very promising when a scaffold is applied to build up tissue-like structures in vitro by the cells culture. β-TCP is a non-toxic and bioabsorbable ceramics which have been clinically used. In this study, it is used to combine the scaffold for the mechanical reinforcement, which is essential to maintain the porous structure of scaffold in cell culture [25]. The porous structure enables cells to give a steric space for their proliferation and differentiation in the scaffold as well as the oxygen and nutrient supply.

As a non-viral carrier of gene transfection, spermine-introduced pullulan (spermine-pullulan) has been explored [26]. This carrier showed a high efficiency of plasmid DNA transfection for BMSC because of an enhanced internalization of plasmid DNA into cells due to the efficient interaction of sugar-residues in pullulan with the asialoglycoprotein receptor of cells [27].

The objective of study is to evaluate the feasibility of gelatin and β-TCP

scaffolds containing the complex of spermine-pullulan and plasmid DNA in the gene transfection for BMSC. To improve the gene transfection efficiency for BMSC in the scaffold, the way to combine the complex with the scaffold was contrived. To improve the extent of complex combination, the scaffold was treated with succinylated gelatin derivatives of negative charge (suc-gel). The suc-gel treatment will enhance the combination of complex, resulting in the increased gene transfection. The 3D gelatin/β-TCP scaffolds were prepared by different methods of suc-gel treatment, and the effect of scaffold type on the gene transfection for BMSC was evaluated. We examine the profile of plasmid DNA from scaffolds containing the complexes and compare with that of gene transfection.

2. Materials and methods

2.1. Materials

A gelatin sample with an isoelectric point (IEP) of 9.0, prepared by an acid process of porcine skin, that with an IEP of 5.0, prepared by a base process of bovine bone, and succinylated gelatin were kindly supplied from Nitta Gelatin Co. (Osaka, Japan). β-TCP granules (average size = 2.9 μm) were obtained from Taihei Chemical Industries, Nara, Japan. Pullulan with a weight-average molecular weight of 47,300 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Spermine was purchased from Sigma Chemical Co., St. Louis, MO. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

2.2. Plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI). The plasmid DNA was multiplied in E. coli (strain DH5a) and purified using QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. The yield and purity of the plasmid DNA were evaluated using ultraviolet spectroscopy (DU® 800 Spectrophotometer, Beckman Coulter, Inc., Fullerton, CA). The absorbance ratio at wavelengths of 260 to 280 nm for the plasmid DNA solution was measured to be between 1.8 and 2.0.

2.3. Preparatiom of cationized pullulan derivatives

Spermine was introduced to the hydroxyl groups of pullulan by a N,N′-carbonyldiimidazole (CDI) activation method [28]. Dehydrated dimethyl sulfoxide (50 ml) containing 50 mg of pullulan was mixed with 263 mg of CDI and subsequently 1.87 g of spermine. Following agitation with a magnetic stirrer at 37 °C for 24 hr, the reaction mixture was dialyzed against ultra-pure double-distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight of dialysis membrane is 12,000–14,000, Viskase Companies, Inc, Willowbrook, Illinois). Then, the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine–pullulan). The spermine introduction, expressed by the molar percentage of spermine introduced to the hydroxyl groups of pullulan, was 11.8 mole/mole%, when determined by the conventional elemental analysis.

2.4. Preparation of polyion complexes of plasmid DNA and spermine-pullulan

The spermine-pullulan aqueous solution (223 μg/ml) was mixed with

phosphate-buffered saline solution (PBS, 10 mM, pH 7.4) containing 100 μg/ml of plasmid DNA, followed by leaving for 15 min at room temperature to obtain the polyion complex.

2.5. Preparation of sponge scaffolds of gelatin and β-TCP for gene transfection

Gelatin scaffolds of sponge type incorporating β-TCP were prepared as previously reported [25]. Briefly, 3 wt% aqueous solution of gelatin containing different amounts of β-TCP was mixed at 5,000 rpm at 37 °C for 3 min by using a homogenizer (ED-12, Nihonseiki Co., Tokyo, Japan). After aqueous solution of glutaraldehyde (GA) was added to the mixed solution, the mixed β-TCP and gelatin solution was further agitated for 15 sec by the homogenizer. The resulting bubbled solution was cast into a polypropylene dish, followed by leaving at 4 °C for 12 hr for gelatin crosslinking. Then, the crosslinked gelatin scaffold of sponges was freeze-dried, and then placed in 100 mM aqueous glycine solution at 37 °C for 1 hr to block the residual aldehyde groups of glutaraldehyde, thoroughly washed with DDW, and freeze-dried, followed by cut into discs with 6 mm in diameter and 3 mm thickness. The average pore size, porosity, compression modulus of the scaffolds were measured as previously reported [25].

The scaffold discs were immersed in the aqueous solution of succinylated gelatin (suc-gel, 100 μl) at 37 °C for 24 hr, freeze-dried, and dehydrothermally crosslinked under vacuum at 140 °C for 32 hr in a vacuum oven (Sato vacuum machinery industrial Co., LTD., Tokyo, Japan). After the removal of excess suc-gel by rinsing with PBS, 100 μl of polyion complex solution was added to the suc-gel-treated scaffold, followed by incubation at 37 °C for 2 hr to allow the complex to associate in the scaffold.

2.6. Cell culture

BMSC were isolated from the bone shaft of femurs and tibias of 3-wk-old male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) according to the technique reported by Lennon et al [29]. Briefly, both the ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 ml of alpha minimum essential medium (αMEM) supplemented with 15 vol% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 U/ml). The cell suspension (5 ml) was placed into two 25 cm^2 flasks (Iwaki Glass, Funabashi, Chiba, Japan) and cultured at 37 °C in a 95% air-5% carbon dioxide atmosphere. The medium was changed 2 and 3 days after culture and thereafter every 3 days. When the cells of the first passage became sub-confluent, usually 7 to 10 days after seeding, the cells were detached from the flask using treatment for 5 min at 37 °C with PBS solution containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid (Sigma-aldrich, Inc., St Louis, MO). Cells were normally subcultured at a density of 2×10^4 cells/cm^2. Second-passaged cells in a sub-confluence condition were used for all the experiments.

2.7. Cell culture in scaffolds for gene transfection

BMSC were seeded into the scaffolds by an agitated seeding method reported previously [30]. Briefly, 350 μl of cell suspension (2×10^6 cells/ml) and one scaffold were placed in 5-ml tube with 12 mm of inner diameter (IWAKI Glass Co. Ltd., Chiba, Japan) and agitated on an orbital shaker (Bellco Glass, Inc., Vineland, NJ) at 300 rpm for 6 hr. The cell-seeded scaffolds were spired by a needle and immobilized in the spinner flasks (Bellco Glass, Inc., Vineland, NJ). The spinner flask was filled with 150

ml of culture medium and the medium was stirred at the rate of 50 rpm. The medium was totally changed every 3 days. At different time intervals, the scaffold with cells was washed with PBS once, and stored at -80 °C for further experiments.

2.8. Evaluation of Suc-gel amount remaining in scaffolds

The amount of suc-gel remaining in the scaffolds was evaluated by use of suc-gel labeled with rhodamine B (suc-rhoB). The suc-rhoB was prepared according to the procedure reported previously [28]. Briefly, dehydrated DMSO (25 ml) containing 1 mg/ml of rhodamine B isothiocyanate was gently added to 500 ml of sodium carbonate buffer (0.1 M, pH 9.0) containing 2 mg/ml of acidic gelatin. After gentle stirring for 10 hr at 4 °C, 25 ml of ammonium chloride aqueous solution (1.1 M) was added to the mixture to stop the labeling reaction with gentle stirring for 2 hr at 25 °C. Acidic gelatin labeled with rhodamine B was purified by dialysis against DDW for 2 days with a dialysis membrane with the cut-off molecular weight of 12,000–14,000, and then freeze-dried. The rhodamine-labeled gelatin was dissolved dehydrated DMSO, while succinic anhydride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the DMSO solution, followed by leaving for 8 hr at 25°C. The amount of primary amino groups remaining of suc-rhoB was determined by the trinitrobenzene sulfonic acid (TNBS) method to experimentally confirm the 100% conversion to the amino groups of gelatin to carboxyl ones.

Scaffolds treated with rhodamine-labeled suc-gel by different methods were rinsed with PBS or αMEM without phenol red containing 10 vol% of FCS. Then, the scaffolds were enzymatically degraded by incubating at 37 °C for 1 hr in 150 μl of PBS containing 0.1 w/v% of trypsin (Sigma-aldrich, Inc., St Louis, MO). After separation of

β-TCP granules by centrifugation, the fluorescent intensity of supernatant was measured at the excitation and emittion wavelengths of 544 and 590 nm, respectively. A calibration curve was prepared for the scaffolds treated with pre-determined concentrations of rhodamine-labeled suc-gel to evaluate the amount of suc-gel associated in the scaffold.

2.9. Evaluation of polyion complexes release from gelatin/β-TCP scaffolds

The release of spermine-pullulan and plasmid DNA complexes was evaluated by use of ^{125}I-labeled plasmid DNA. Briefly, the plasmid DNA was labeled with ^{125}I according to the method reported previously [20]. Similar procedure was done to prepare the scaffolds containing the complex other than using ^{125}I-labeled plasmid DNA. After the cell culture of scaffold, the amount of plasmid DNA immobilized and released was determined by assessing the radioactivity of scaffolds remaining.

2.10. Luciferase assay

Scaffold with cells was immersed in 200 μl of cell culture lysis reagent (Promega Corp., Madison, WI) in a micro reaction tube, then crushed using Tissue Lyser (Retsch GmbH & Co. KG, Haan, Germany) at room temperature with for 10 min. The debris of cells and scaffold were separated by centrifugation (17,800 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI) was added to 20 μl of supernatant, and the relative light unit (RLU) of the sample was determined using a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan).

The total protein of cells in each scaffold was determined using a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to the manufacturers'

instructions. The amount of scaffold protein was subtracted with the appropriate background. The RLU was divided by the protein amount to normalize the number of cells on the luciferase activity. Each experimental group was carried out 3 times independently.

2.11. Statistical analysis

Data were expressed as means ± standard deviations. Data were analyzed using Tukey-Kramer paired comparison test, while the significance was accepted at $p<0.05$.

3. Results

3.1. Preparation of gelatin/β-TCP scaffolds

Table 1 shows the characteristics of scaffolds prepared at different amounts of β-TCP and GA. The average pore size was about 200 μm, irrespective of the preparing condition of scaffolds.

3.2. Gene transfection ability of scaffolds

Figure 1 shows that the gene transfection level of cells cultured in the gelatin/β-TCP scaffolds prepared by different methods. When the β-TCP content was 75% and higher, the gene expression level of cells significantly decreased. The maximum gene expression was observed for the scaffolds prepared at the GA concentrations of 26 and 52 moles/moles. The gene expression level of cells depended on the preparation method of scaffolds and the highest level was observed for the scaffold prepared by the method C. The gene transfection was influenced by the concentration of suc-gel treated with the scaffolds and became maximum at the concentrations of 50 and 500 μg / scaffold.

3.3. Remaining of suc-gel in gelatin/β-TCP scaffolds

Figure 2 shows the amount of suc-gel remaining in the scaffolds prepared by different methods and different amounts of suc-gel treated after rinsing of PBS with or without the culture medium. A large amount of suc-gel was detached from scaffold by rinsing with the culture medium for scaffolds prepared by the method A, in contrast to those prepared by the methods B and C. There was significant difference in the amount of suc-gel remaining in scaffolds treated between the methods B and C.

The percentage of suc-gel remaining in scaffolds depended on the amount of suc-gel added to scaffolds. As the amount of suc-gel treated increased, the percentage of suc-gel detached from scaffolds also increased.

3.4. The amount of plasmid DNA remaining and release profile

Figure 3 shows the amount of plasmid DNA immobilized in scaffolds prepared by different methods of suc-gel treatment or at various amounts of suc-gel. The amount of plasmid DNA remaining was influenced by the method of suc-gel treatment and the amount of suc-gel treated, and increased at the suc-gel concentration of 5 and 50 μg.

Figure 4 shows the profiles of complex release from scaffolds. Irrespective of the initial amount of plasmid DNA immobilized in scaffolds, the plasmid DNA was released.

3.5 Gene transfection of gelatin/β-TCP scaffolds containing complex

Figure 5 shows the relationship between the gene expression level of MSC cultured in gelatin/β-TCP scaffolds prepared different methods and the amount of

plasmid DNA released. The gene transfection depended on the preparation method of scaffolds containing the complex and the amount of suc-gel treated. The level of gene expression was in good accordance with that of plasmid DNA released.

Figure 6 shows the proliferation of cells in scaffolds prepared at different amounts of suc-gel added. On day 1 and 7, there is no significant difference in the cell number among the experimental groups.

4. Discussion

The present study clearly demonstrates that the plasmid DNA transfection for MSC was successfully achieved by their culturing in the gelatin/β-TCP 3D scaffolds containing the plasmid DNA complexed with spermine-pullulan. The level of plasmid DNA transfection depended on the method of scaffolds preparation and the order was in good accordance with that of plasmid DNA released. It is possible that cells were transfected by the complex released from the scaffold in culture.

In this culture system, BMSC were cultured and transfected on the inner surface of 3D scaffolds which is immobilized with the plasmid DNA complexes. Considering the positioning of cells and the complex, it is likely that the complex always exists near the cells to be transfected. It is highly conceivable that both the continuous exposure of complex to cells and the confirm attachment between cells and the substrate which minimizes the serum influence on the transfection activity of complex, result in such an enhanced plasmid DNA transfection. This transfection system, called the reverse transfection, has been reported () and the transfection efficiency was high compared with that of conventional method where the complex is added into the medium for transfection culture of cells. This study is to demonstrate that

the reverse transfection system was applicable to the 3D scaffold of cell culture.

The level of gene expression for MSC on the 3D scaffold was lower than that of the 2D scaffold (data not shown). Considering the fact that the cells conditions are strongly influenced by their extracellular environment, it is understandable that the 3D environment is suitable to cells for gene transfection. Further investigation is needed to understand the mechanism of superior gene transfection of 3D scaffolds to 2D ones.

The gene expression level of cells cultured in scaffolds was influenced by the amount of β-TCP and GA in the scaffolds (**Figure 1**). On the other hand, the cell proliferation was also affected by the preparing procedure of scaffolds (data not shown). As the amount of β-TCP and GA increased, the number of cells proliferated in scaffolds increased. It is highly conceivable that the increased amount enhanced the mechanical strength of scaffolds, which can contain the porous structure necessary for cell proliferation. As the result, the cell proliferation was enhanced and consequently the gene transfection was increased. However, at the higher amounts of β-TCP and GA, the level decreased. The reason is not clear at present although the scaffold stiffness may affect the gene transfection.

The amount of suc-gel remaining in scaffolds increased as that of suc-gel treated became high (**Figure 2a**). This result can be explained by the suc-gel detachment. The detachment of suc-gel from scaffolds may be due to replacement of suc-gel by proteins present in FCS since the PBS rinsing did not cause the detachment. Suc-gel treated by the method A would bind to the scaffolds by the interaction weaker, such as the electrostatic interaction, than that with the methods B and C. By freeze-drying (method B), the interaction between the suc-gel and scaffold will become stronger. This is because other interaction forces including the hydrophobic interaction

were added. By heating under vacuum (method C), chemical bonds can be formed between the carboxyl groups of suc-gel and the amino groups or hydroxyl groups of gelatin through dehydrothermal reaction. Taken together, the dehydrothermal process would increase the interaction between the suc-gel and the gelatin of scaffold, which results in high resistance of suc-gel against FCS replacement and the consequent increase in the amount of suc-gel remaining in the scaffolds. When the scaffolds were treated with more than 500 μg of suc-gel, the percentage of suc-gel detached from scaffolds increased **(Figure 2b)**. This can be explained by an increase in the amount of suc-gel that is not interacted with the components of scaffolds.

The suc-gel of negative charge can strong bind to the gelatin molecules of scaffolds. This enables the complex of positive charge to increase the amount associated with the scaffold. Comparing the amount of plasmid DNA immobilized with that of suc-gel remaining (**Figures 2 and 3**), it is clear that the scaffolds with less detachable suc-gel could associate higher amount of plasmid DNA. This relationship clearly indicates that the suc-gel mediated the association of polyion complexes to the scaffold. This was experimentally confirmed by the result of plasmid DNA associated at the concentrations ranging from 0 to 50 μg. At the higher amounts of suc-gel, it is possible that suc-gel can be detached easily from the scaffold together with polyion complex, resulting in suppressed amount of plasmid DNA immobilized.

When compared among the scaffolds with different suc-gel treatments, it was revealed that the amount of plasmid DNA immobilized corresponded to that of plasmid DNA released (**Figures 3a and 5a**). The finding suggests that the interaction strength between the plasmid DNA and the suc-gel treated is similar, irrespective of suc-gel treatment method.

There was good correlation between the amount of plasmid DNA released and the gene expression level (**Figure 5**). This can be explained from the viewpoint of plasmid DNA release. The complex would be released into culture medium during the cell culture, and then the complex would be taken up by the cells cultured in the scaffold. The profile of gene expression could be regulated by changing that of complexes released. This trend was also observed for 2D substrates. The complex released from the substrate surface was taken up by cells and the consequent gene transfection was observed [4].

Although the gene expression level of cells was influenced by the amount of β-TCP or GA in the scaffolds, there was no difference in the amount of plasmid DNA released from the scaffolds (**Figure 4**). This finding suggests that the extracellular environment, such as elasticity or hydrophilicity, affects the culture conditions of cells in scaffolds, resulting in changed level of gene expression.

There have been reported on 3D scaffolds containing plasmid DNA [2, 5, 6, 8]. The scaffolds are basically prepared by simultaneously mixing a plasmid DNA or the complex with the scaffold material. In the system, since the plasmid DNA or the complex is homogenously in the bulk of scaffold, the release behavior of plasmid DNA or the complex is governed by the material property of scaffold. On the other hand, in the case of the present gelatin/β-TCP 3D scaffold, the plasmid DNA complexed with spermine-pullulan is treated to immobilize after the preparation of gelatin/β-TCP scaffold itself. Considering the molecular size of gelatin mesh in the scaffold and complex, it is conceivable that the complex cannot penetrate into the inside of scaffold matrix and is localized only in the surface of scaffold. Therefore, the release profile of complexes is governed only by the interaction strength between the complex and

scaffold material. Since the degradability of scaffold itself and the release of complex can be separately regulated, it is highly expected that in the present scaffold system, the regulation of complex release in the 3D scaffold can be readily controlled. The complex release system governed by the interaction with suc-gel can be regulated and applied for other scaffolds which can bind the suc-gel. This system is applicable for the scaffold of basic cell biology and tissue regeneration.

Gelatin sponges were used as biodegradable 3D scaffolds for plasmid DNA transfection. It has been reported that growth factors, nucleic acids, and low-molecular-weight drugs, could be released from the gelatin scaffold in the controlled fashion. Therefore, the 3D scaffold of gelatin for plasmid DNA transfection can be combined with other bioactive substances. This combination will be effective in design and preparation of cell scaffolds with biological functions which can open a new field of cell biology and regenerative medical therapy.

5. Conclusions

The plasmid DNA transfection for MSC was successfully achieved by their culturing in the gelatin/β-TCP 3D scaffolds containing the plasmid DNA complexed with spermine-pullulan. The level of plasmid DNA transfection depended on the method of scaffolds preparation and the order was in good accordance with that of plasmid DNA released.

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Figure Legends

Figure 1. The gene expression level of cells after culture in gelatin/β-TCP scaffolds containing plasmid DNA. The amount of plasmid DNA added was 5 μg/scaffold. The scaffolds were prepared in different conditions: (a) The β-TCP content, (b) the amount of GA added, (c) the method of suc-gel treatment, and (d) the amount of suc-gel added. (a) The amounts of GA and suc-gel added were 52 mole/mole gelatin and 50 μg/scaffold, respectively. The scaffolds were prepared by the method C. *, $p < 0.05$; significant against the gene expression level of cells cultured in the scaffolds with 0% of β-TCP. (b) The β-TCP content and the amount of suc-gel added were 50 wt% and 50 μg/scaffold, respectively. The scaffolds were prepared by the method C. *, $p < 0.05$; significant against the gene expression level of cells cultured in the scaffolds with 208 mole/mole gelatin of GA. (c) The β-TCP content was 50 wt%. The amounts of GA and suc-gel added were 52 mole/mole gelatin and 50 μg/scaffold, respectively. The method A: scaffolds treated with suc-gel aqueous solution at 37 °C for 24 hr, the method B: scaffolds freeze-dried following the method A, and the method C: scaffolds treated at 140°C for 32 hr under vacuum following the method B.*, $p < 0.05$; significant against the gene expression level of cells cultured in other methods. (d) The β-TCP content and the amount of GA added were 50 wt% and 52 mole/mole gelatin, respectively. The scaffolds were prepared by the method C. *, $p < 0.05$; significant against the gene expression level of cells cultured in other groups.

Figure 2. (a) The amount of suc-gel remaining in gelatin/β-TCP scaffolds prepared by different methods: (A) scaffolds treated with suc-gel aqueous solution at 37 °C for 24 hr, (B) scaffolds freeze-dried following the method A, and (C) scaffolds treated at 140°C

for 32 hr under vacuum following the method B. The β-TCP content was 50 wt%. The amounts of GA and suc-gel added were 52 mole/mole gelatin and 50 μg/scaffold, respectively. The amount of suc-gel remaining was determined after rinsing the scaffolds with PBS (solid columns) or the culture medium (open columns). *****, $P<0.05$: significant against the amount of suc-gel remaining in scaffolds prepared by the method A at the corresponding rinsing procedure. †, $P<0.05$: significant against the amount of suc-gel remaining in scaffolds prepared by the method B at the corresponding rinsing procedure.

(b) Percentage of suc-gel remaining in gelatin/β-TCP scaffolds treated with different amounts of suc-gel. The β-TCP content and the amount of GA added were 50 wt% and 52 mole/mole gelatin, respectively. The scaffolds were prepared by the method C. *****, $P<0.05$: significant against the amount of suc-gel remaining in scaffolds prepared by the method A at the corresponding rinsing procedure. †, $P<0.05$: significant against the amount of suc-gel remaining in scaffolds prepared by the method B at the corresponding rinsing procedure.

Figure 3. The amount of pDNA remaining in gelatin/β-TCP scaffolds prepared by different methods (a), or those treated with different amounts of suc-gel (b) after cell seeding: (A) scaffolds treated with suc-gel aqueous solution at 37 °C for 24 hr, (B) scaffolds freeze-dried following the method A, and (C) scaffolds treated at 140°C for 32 hr under vacuum following the method B. The amount of plasmid DNA added was 5 μg/scaffold. (a) The β-TCP content was 50 wt%. The amounts of GA and suc-gel added were 52 mole/mole gelatin and 50 μg/scaffold, respectively. *****, $P<0.05$: significant against the amount of plasmid DNA remaining in scaffolds without suc-gel treatment (0

μg / scaffold). (b) The β-TCP content and the amount of GA added were 50 wt% and 52 mole/mole gelatin, respectively. The scaffolds were prepared by the method C. *****, $P<0.05$: significant against the amount of plasmid DNA remaining in scaffolds without suc-gel treatment (0 μg / scaffold). †, $P<0.05$: significant against the amount of plasmid DNA remaining in scaffolds treated with 5 μg/scaffold of suc-gel.

Figure 4. The time profiles of plasmid DNA release from gelatin/β-TCP scaffolds prepared by different methods (a), or those treated with different amounts of suc-gel (b) during cell culture. The amount of plasmid DNA added was 5 μg/scaffold. (a) (○) scaffolds without suc-gel treatment, (△) scaffolds treated with suc-gel aqueous solution at 37 °C for 24 hr (method A), (□) scaffolds freeze-dried following the method A (method B), and (●) scaffolds treated at 140°C for 32 hr under vacuum following the method B (method C). The β-TCP content was 50 wt%. The amounts of GA and suc-gel added were 52 mole/mole gelatin and 50 μg/scaffold, respectively. (b) scaffolds treated with 0 (○), 5 (△), 50 (□), 500 (●), and 1500 μg/scaffold of suc-gel (▲). The β-TCP content and the amount of GA added were 50 wt% and 52 mole/mole gelatin, respectively. The scaffolds were prepared by the method C.

Figure 5. Relationship between the gene expression level of MSC cultured in gelatin/β-TCP scaffolds prepared by different methods (a), or those treated with different amounts of suc-gel (b) for 3 days (○) and the amount of plasmid DNA released from the scaffolds during 3 days of cell culture (△).

Figure 6. The number of MSC cultured for 1 (open columns) and 7 days (solid

columns) in gelatin/β-TCP scaffolds treated with different amounts of suc-gel. The β-TCP content and the amount of GA added were 50 wt% and 52 mole/mole gelatin, respectively. The scaffolds were prepared by the method C.

Table 1. Characteristics of β-TCP/gelatin scaffolds used.

β-TCP (wt%)	GA (mole/mole gelatin)	Average pore size (μm)	Porosity (%)	Water content ((w/w)%)
0	52	172.3 ± 50.2	98.5 ± 0.1	97.6 ± 0.07
12.5		189.0 ± 35.6	98.4 ± 0.0	
25		179.2 ± 36.4	97.9 ± 0.0	
50		191.0 ± 55.0	97.9 ± 0.1	
75		191.9 ± 29.9	96.8 ± 0.3	
90		172.9 ± 40.3	93.6 ± 0.3	
50	13	183.5 ± 38.4	97.8 ± 0.2	98.2 ± 0.09
	26	185.3 ± 36.3	97.5 ± 0.5	97.8 ± 0.09
	52	191.0 ± 55.0	97.9 ± 0.1	97.6 ± 0.07
	104	208.0 ± 35.9	97.9 ± 0.0	97.3 ± 0.10
	208	187.1 ± 31.6	97.9 ± 0.1	97.3 ± 0.07

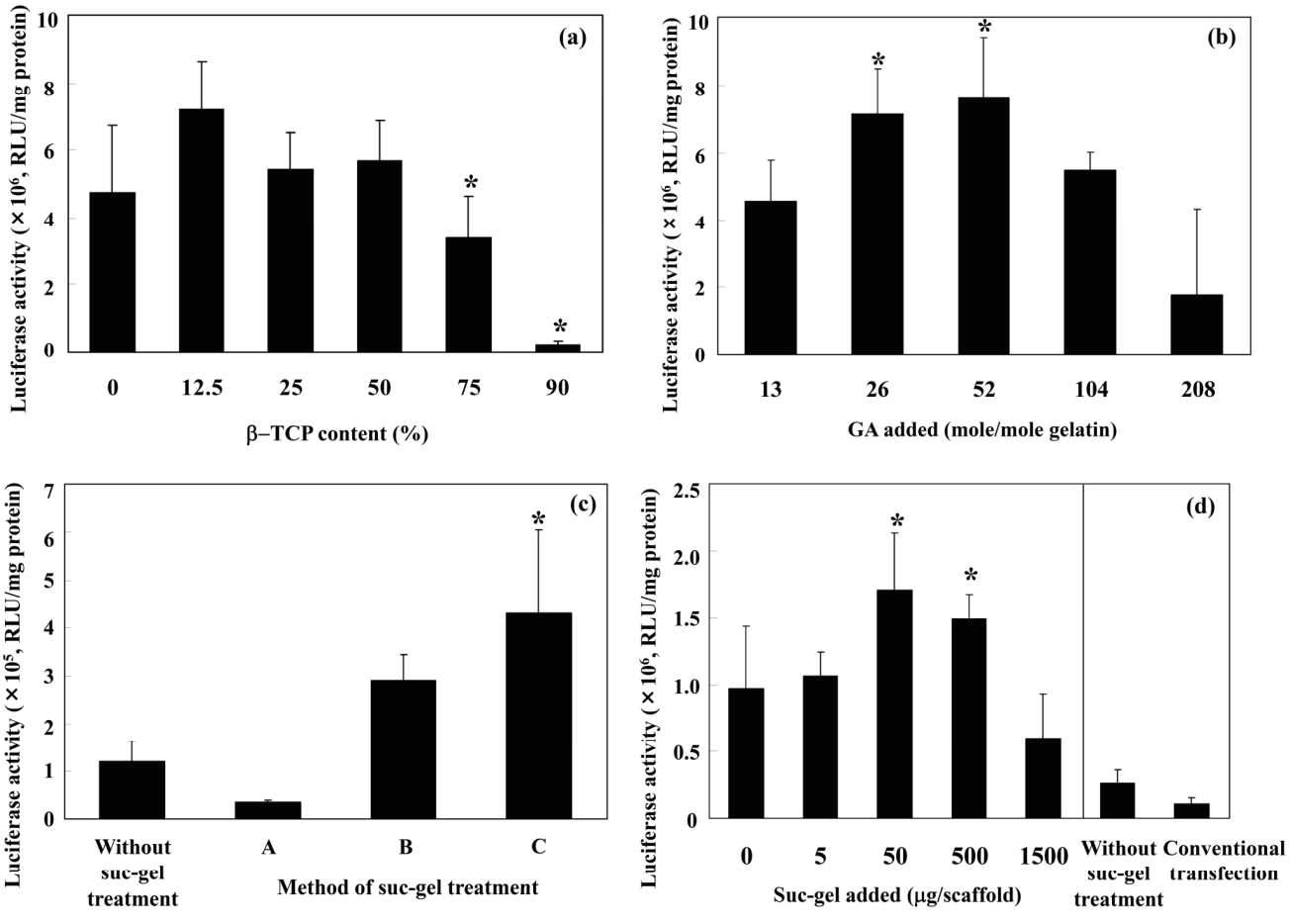


Figure 1. Kido et al.

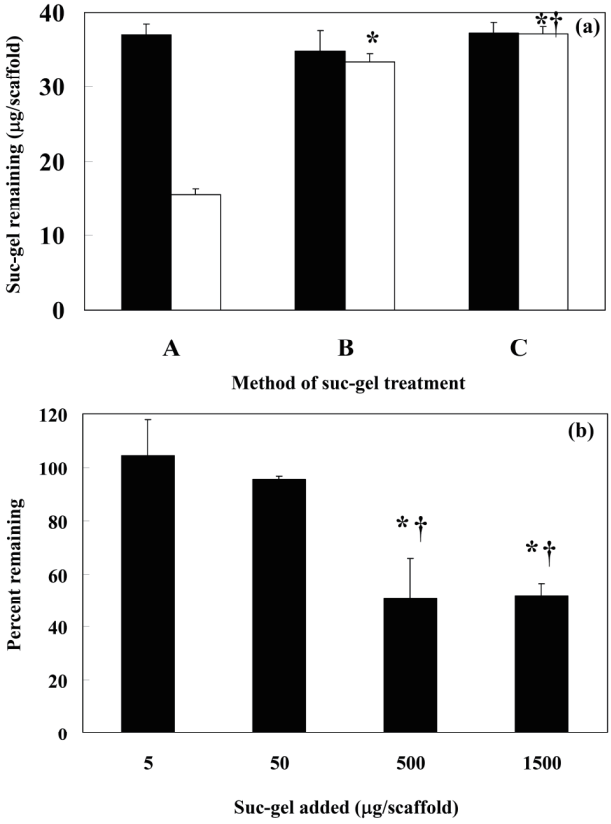
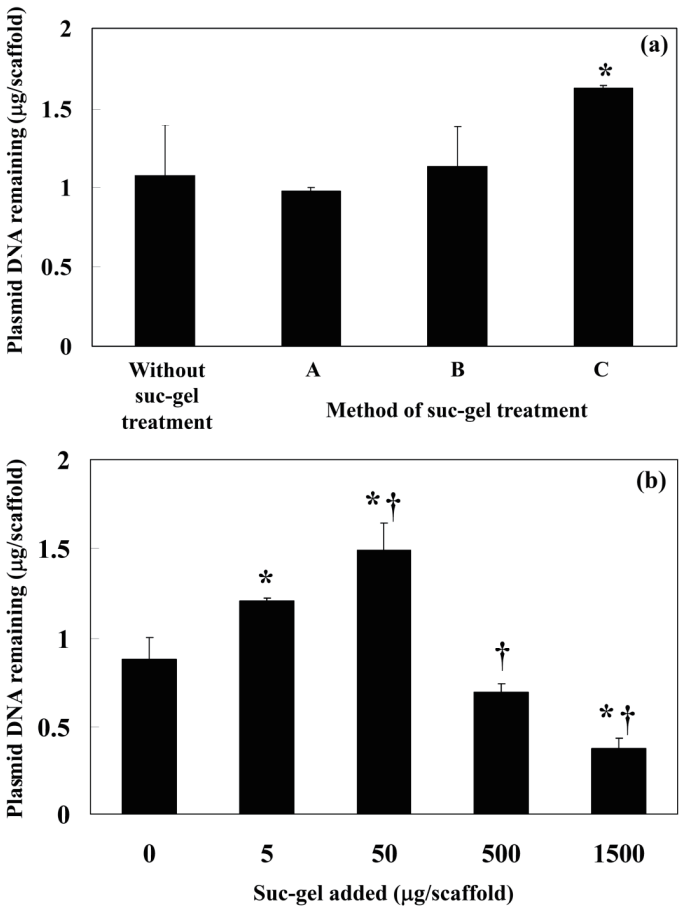


Figure 2. Kido et al.



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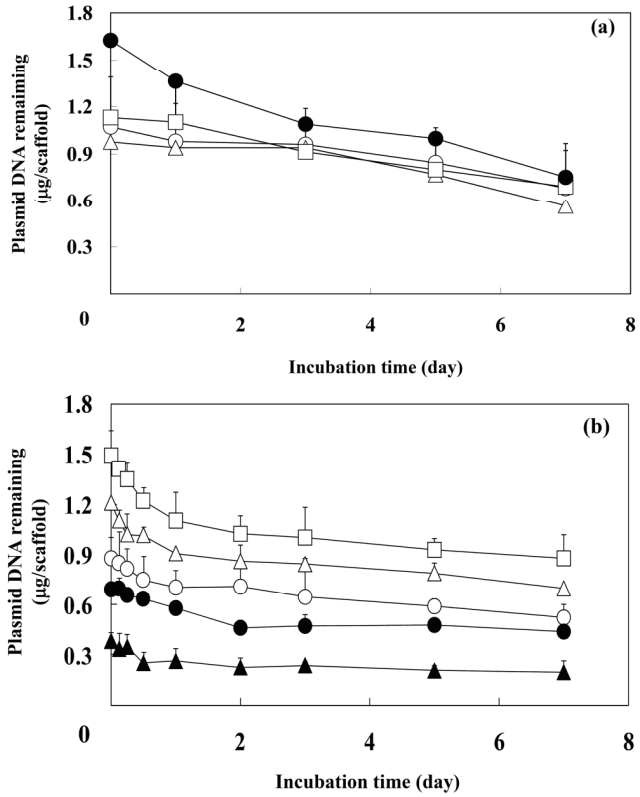

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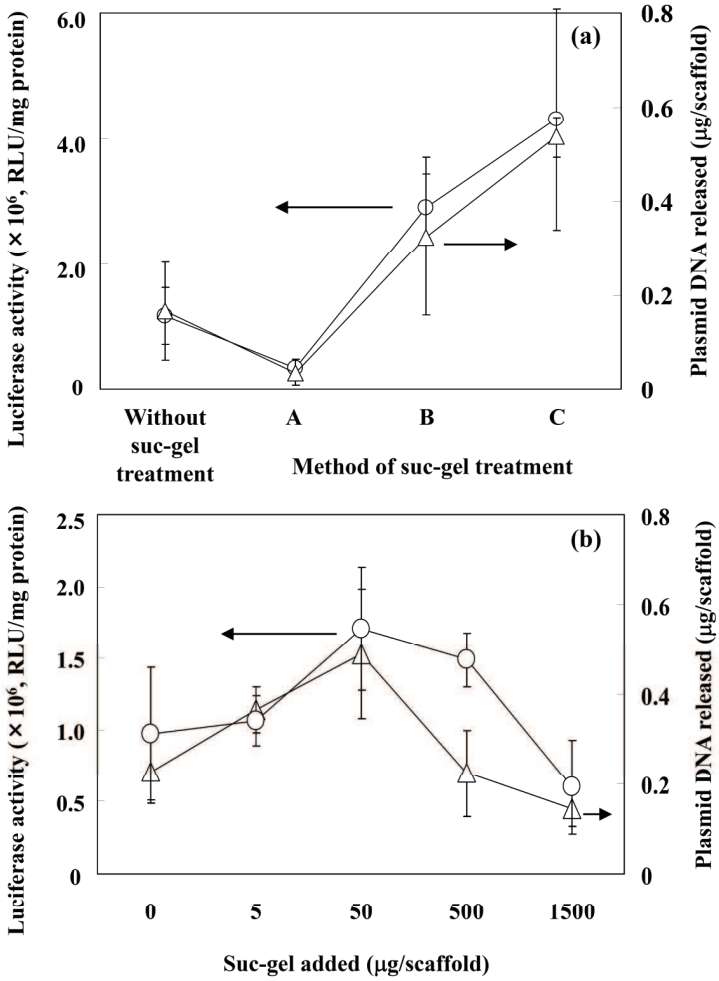

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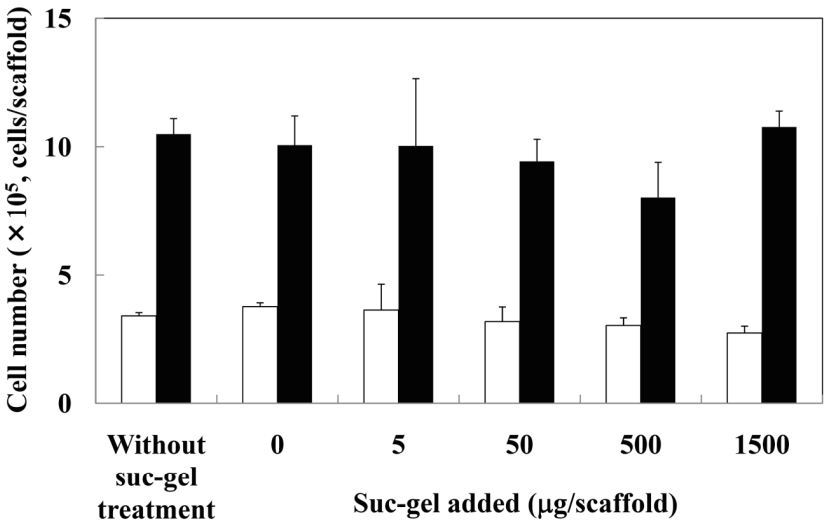

Figure 6. Kido et al.